Expression of Amphiregulin Is Regulated in Cultured Human Keratinocytes and in Developing Fetal Skin

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Previous studies have indicated that amphiregulin is a major autocrine factor for human keratinocytes. To evaluate the possibilities that amphiregulin could function in fetal skin morphogenesis and contribute to the growth regulation of epidermis, immunostaining with a specific anti-amphiregulin monoclonal antibody was observed at different stages of fetal skin development, and the results were compared with neonatal and adult skin specimens and cultured neonatal keratinocytes. Immunoreactive amphiregulin was readily detected in the periderm and basal epidermal layers of embryonic epidermis but became gradually less detectable in the periderm concurrent with an increase in staining of the spinous layer as it developed during the fetal period. Basal and spinous keratinocyte expression of amphiregulin was predominantly cytoplasmic, but with punctate nuclear foci, and this pattern persisted into the neonatal period. At all developmental stages, epithelial and mesenchymal cells of the follicle were reactive, often in a nuclear pattern. Dermal mesenchymal cells were increasingly reactive in late fetal skin, but the staining decreased postnatally. In adult skin only randomly scattered nuclei of spinous keratinocytes and follicular structures such as the inner root sheath were stained. Examination by scanning laser confocal microscopy of cultured neonatal keratinocytes showed a nonrandom distribution of amphiregulin to the peripheral cytoplasm and plasma membranes at the outer perimeter of cell colonies, with much less reactivity of apposed keratinocyte membranes at interior sites. Nuclei were heterogeneously stained. Amphiregulin reactivity declined at higher cell densities. These data indicate that expression of amphiregulin is regulated *in vitro* and developmentally during cutaneous morphogenesis. ***Key words: epidermal growth factor/epidermal growth factor receptor. J Invest Dermatol 105:802–809, 1995***

Among the epidermal growth factor family of structurally homologous proteins is a unique factor with binding affinity for heparin-like glycosaminoglycans known as amphiregulin [1–5]. First isolated from the conditioned medium of mitogenically-stimulated human mammary epithelial cells [6,7], amphiregulin is the product of a single-copy gene that maps to chromosome 4q [1]. The gene encodes a transmembrane precursor protein, the extracellular domain of which is proteolytically processed into a mature cytokine that interacts specifically with the 170-kDa epidermal growth factor receptor [8]. The amino terminus of the mature factor contains two strongly basic loci that have been postulated to function as recognition sequences for nuclear targeting [1]. Reports of the nuclear localization of amphiregulin, in fact, indicate a direct role in the regulation of gene expression [9–12], in addition to secondary signaling events that occur after its binding to specific membrane receptors.

Previous studies have indicated that amphiregulin contributes significantly to the autocrine growth of cultured human keratinocytes and that its biologic effects are regulated by cellular glycosaminoglycans [3,5]. Additional regulatory mechanisms are suggested by the observation that exogenous epidermal growth factor and transforming growth factor alpha stimulate the transcription of the amphiregulin gene [13,14]. The up-regulated transcription observed in certain hyperproliferative skin conditions [15–17] further indicates that increased expression of amphiregulin may contribute to the pathophysiology of skin disease. Its precise physiologic roles in the regulation of keratinocyte growth under normal and pathologic conditions and during morphogenesis of the skin remain to be elucidated. In this report, we describe the immunolocalization of amphiregulin during fetal skin development and compare these patterns with those observed in neonatal and adult skin, as well as in cultured neonatal keratinocytes.

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MATERIALS AND METHODS

Keratinocyte Culture Primary keratinocyte cultures were established from human neonatal foreskins, and the cells were used at the second to fourth passage levels [5]. Chamber slides (Falcon, Becton Dickinson, Lincoln Park, NJ) were inoculated with keratinocytes at 1–3 $\times$ 10^3 cells/cm^2 and cultivated for 24 h in keratinocyte basal medium (Clonetics, San Diego, CA) with 5 $\times$ 10^{-7} M hydrocortisone, 10^{-4} M ethanolamine, gentamycin, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.2% bovine pituitary extract (hereafter referred to as complete keratinocyte growth medium, cKGM). Thereafter, experimental medium, consisting of cKGM without epidermal growth factor or bovine pituitary extract, was

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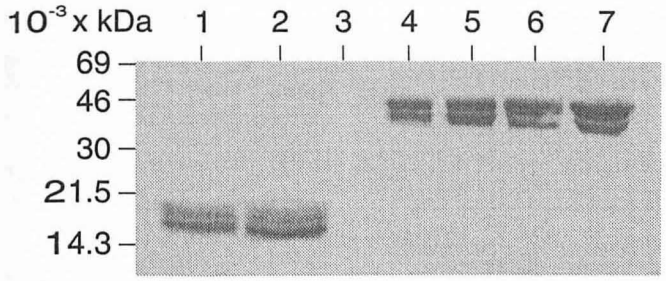


Figure 1. Specificity of the 6R1C anti-amphiregulin antibody established by Western immunoblotting of keratinocyte extracts and conditioned media. The molecular weights of the standard markers are indicated in the left margin. The *first* and *second lanes* represent one and two times applications of material from keratinocyte conditioned medium. The keratinocyte extracts are analyzed in *lanes 4–7* over the concentration range of 5, 8, 12, and 15 μg of total protein, respectively. Detection was by enhanced chemiluminescence (*Materials and Methods*).

added to the chambers for at least 2 d prior to amphiregulin immunolocalization.

Prenatal Skin Specimens Embryonic and fetal skin samples of 7–20 weeks estimated gestational age (EGA) were obtained through the Central Laboratory for Human Embryology at the University of Washington, in accordance with guidelines of the U.S. Department of Health and Human Services and with the approval of the Human Subjects Division of the Institutional Review Board. The embryonic stage (samples of 7–8 weeks EGA) showed the epidermis to consist of basal keratinocytes and periderm. The early fetal samples at 9 weeks exhibited epidermal stratification. Follicular structures were studied in 13–26 weeks EGA samples, corresponding to the onset of follicular morphogenesis. Neonatal and adult skin samples were concurrently evaluated.

Western Immunoblotting The specificity of the anti-amphiregulin antibody, 6R1C, was established by Western immunoblotting of conditioned media and cell extracts from human neonatal keratinocyte cultures. Conditioned serum-free cKGM medium, without epidermal growth factor or bovine pituitary extract, was collected from 13 100-mm tissue culture dishes (Falcon) of keratinocytes in exponential growth. Following dialysis against 0.1 M acetic acid, the material was concentrated in electrophoresis sample buffer. The keratinocyte cell layers were washed twice with chilled phosphate-buffered saline and scraped into 1 ml of cold 20 mM HEPES, pH 7.4, with 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, 1 mM phenylmethyl sulfonyl fluoride, and 20 μg/ml aprotinin. Following incubation on ice for 30 min, the cells were pooled and homogenized. The homogenate was centrifuged for 3 min at 300 × *g* at 4°C, and the supernatant was concentrated into sample buffer. The concentrates from the conditioned media and the cell layer extracts at several loading volumes were resolved electrophoretically by non-reducing sodium dodecylsulfate–polyacrylamide gel electrophoresis along with molecular-weight markers (Amersham, Arlington Heights, IL), using 15% acrylamide gels. The gels were transferred for 1 h at 100 volts to nitrocellulose sheets (Immobilon-NC, Millipore, Bedford, MA), which were blocked with Tris-saline (10 mM Tris base, 0.15 M NaCl, pH 7.6) containing 0.25% gelatin/0.05% NP40 (Tergitol, Sigma, St. Louis, MO)/250 mM EDTA for 1 h at room temperature and incubated with a 1:250 dilution of the primary anti-amphiregulin antibody stock solution (1.45 mg/ml) overnight at 4°C. The blots were washed with buffer three times and incubated with 1:500 secondary, horse anti-mouse antibody conjugated to horseradish peroxidase (Vector, Burlingame, CA) for 60 min at room temperature. Following a wash with 0.25% gelatin/0.05% NP40/0.4% sarcosyl in Tris-saline, immunolocalization was by enhanced chemiluminescence according to the manufacturer (ECL, Amersham) with 1-second exposure times (see **Fig 1**, *Results*).

Immunostaining of Keratinocytes The 6R1C antibody was determined to detect amphiregulin in frozen and methyl Carnoys-fixed tissue samples. Antibody localization was by avidin-biotin peroxidase staining of histologic sections from the prenatal, neonatal, and adult skin samples and by fluorescein immunofluorescence of cultured keratinocytes grown on slide chambers. For immunocytochemistry, 6-micron–thick frozen sections of skin or cultured keratinocytes were fixed with ethanol at −20°C for 5 min, blocked with phosphate-buffered saline, pH 7.5, containing 5% bovine serum albumin, and incubated with either a 1:150 dilution of the primary anti-amphiregulin antibody, or buffer or preimmune mouse serum as negative controls, for 1 h. Separate controls were alternatively fixed in 2% paraformaldehyde and processed concurrently to rule out antigen redistribution from the alcohol fixative. Following three buffer washes of the sections and slide chambers, the secondary biotinylated horse anti-mouse antibody conjugated to biotin (1:200, Vector) and peroxidase-labeled streptavidin (1:200) were sequentially applied for 30 min each at room temperature, and reaction product was visualized with hydrogen peroxide and 3,3′-diaminobenzidine (Sigma, St. Louis, MO) as chromogenic substrate.

For localization by immunofluorescence, neonatal foreskin keratinocytes were cultured standardly on slide chambers. The cell layers were rinsed in phosphate-buffered saline, fixed in ethanol at −20°C, rinsed, blocked with serum, and reacted with 1:150 dilutions of the anti-amphiregulin antibody for 1 h. The secondary antibody (horse anti-mouse conjugated to FITC; Vector) diluted to 1:100 was applied for 30 min. The slide chambers were washed, mounted with Vectashield (Vector), and viewed using a Zeiss Universal microscope equipped with epi-illumination or with a BioRad MRC-600 laser scanning confocal microscope.

RESULTS

Specificity of Anti-Amphiregulin Antibody Established by Western Immunoblotting of Keratinocyte Extracts and Conditioned Media The primary anti-amphiregulin antibody, 6R1C, was a murine monoclonal IgG isolated after immunization with a highly-purified recombinant, secretory (18-kDa) human amphiregulin [5]. The antibody reacts with the carboxyl terminal domain that mediates binding to specific epidermal growth factor receptors [1]. Western immunoblotting with the 6R1C anti-amphiregulin antibody detected one major band migrating with an apparent mass of circa 18 kDa, and a trailing minor band, in keratinocyte-conditioned medium **(Fig 1)** that coincides with soluble, glycosylated amphiregulin [1]. The absence of other significant bands indicates no cross-reactivity with other members of the epidermal growth factor family, such as criptoregulin, that may be secreted by keratinocytes. Immunoblotting of cultured keratinocyte extracts detected a doublet of apparent mass ~45 kDa, which is consistent with the heterogeneously glycosylated, transmembrane amphiregulin precursor [1].

Expression of Amphiregulin Changes During Skin Morphogenesis Immunoperoxidase staining for amphiregulin was performed on fetal skin specimens of 7–20 weeks EGA. The results are summarized in **Table I**. During the embryonic and early fetal stages represented by the specimens from 7–9 weeks EGA, periderm and basal epidermal cells stained for amphiregulin in a predominantly cytoplasmic pattern, but with focal punctate nuclear stain **(Fig 2*A–C*)**. Subepidermal mesenchymal cells were also reactive, although less intensely **(Fig 2*A–C*)**. The negative controls were appropriately nonreactive **(Fig 2*D–F*)**.

During the early fetal period from 12–15 weeks EGA, periderm reactivity declined, basal epidermal cells remained reactive, and intermediate cells of the stratifying epidermis became intensely stained **(Fig 3*A–B*)**. Cells of the hair germ, hair peg, and bulbous hair peg were variably reactive **(Fig 3*A–C,E*)**. There was basal staining of keratinocytes of the hair peg and of cells of the subepidermal zone, the dermal mesenchyme, and the dermal papilla. Many dermal cells exhibited punctate nuclear stain. Microvascular endothelium in the dermis was reactive, as were the medial smooth muscle cells, but not the endothelial cells, of larger subcutaneous blood vessels **(Fig 3*D*)**. For comparison, immunostaining of fetal epidermis for the epidermal growth factor receptor is illustrated in **Fig 4** at 87 d EGA.

At 16–20 weeks, the outer surface and cytoplasm of the periderm were nonreactive, whereas staining was increased in the subjacent keratinocyte layer **(Fig 5*A,B*)**. Keratinocytes in hair follicles were reactive for amphiregulin **(Fig 5*A–E*)**, and connective tissue cells of the dermis showed increased immunostaining compared with the earlier samples. Many dermal cells, including those of the dermal

Table I. Amphiregulin Is Differentially Expressed Within the Skin During Morphogenesis

Structure	Expression Level[a]				
	7–11 weeks[b]	12–15 weeks[b]	16–20 weeks[b]	NBFS[c]	Adult
Periderm	++	+/−	−	NA	NA
Epidermis				Entire	
Basal	++	+	+		−
Suprabasal	NA	++	++		Focal
Follicle					
Germ	NA	++	NA	NA	NA
Peg	NA	++	NA	NA	NA
Bulbous peg	NA	++	NA	NA	NA
Papillae	NA	++	+	ND	ND
Follicle unit	NA	NA	++	+	+/−
Dermis	+	++	++[d]	+	Focal
Blood vessel	NA	+	+	++	+/−

[a] −, nonreactive; +, faint or slight staining; ++, moderate to strong staining.
[b] Estimated gestational age.
[c] NBFS, newborn foreskin; NA, not applicable; ND, not determined.
[d] Staining of a dermal nerve was also observed.

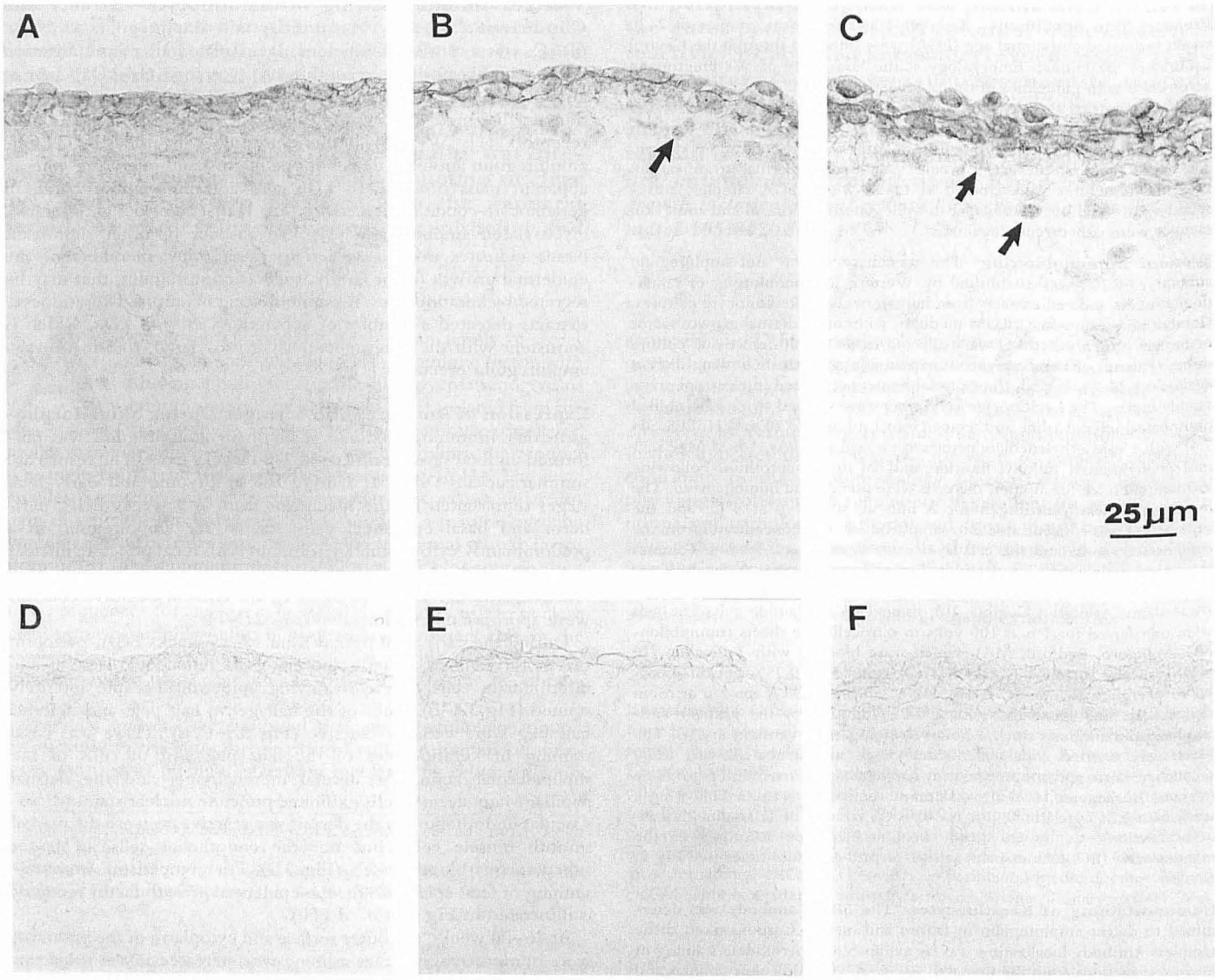


Figure 2. Amphiregulin expression is regulated in fetal skin tissues at 47 d (*A*), 58 d (*B*), and 67 d (*C*) EGA. The two-cell layer epidermis is reactive at 47 d (*A*). Staining is increased at 58 d (*B*), and at 67 d the intermediate cell layer is also reactive (*C*). Dermal cells appear weakly stained, especially proximal to the epidermis (*arrows, B,C*). *D, E, and F* represent the respective negative controls for *A, B, and C*.

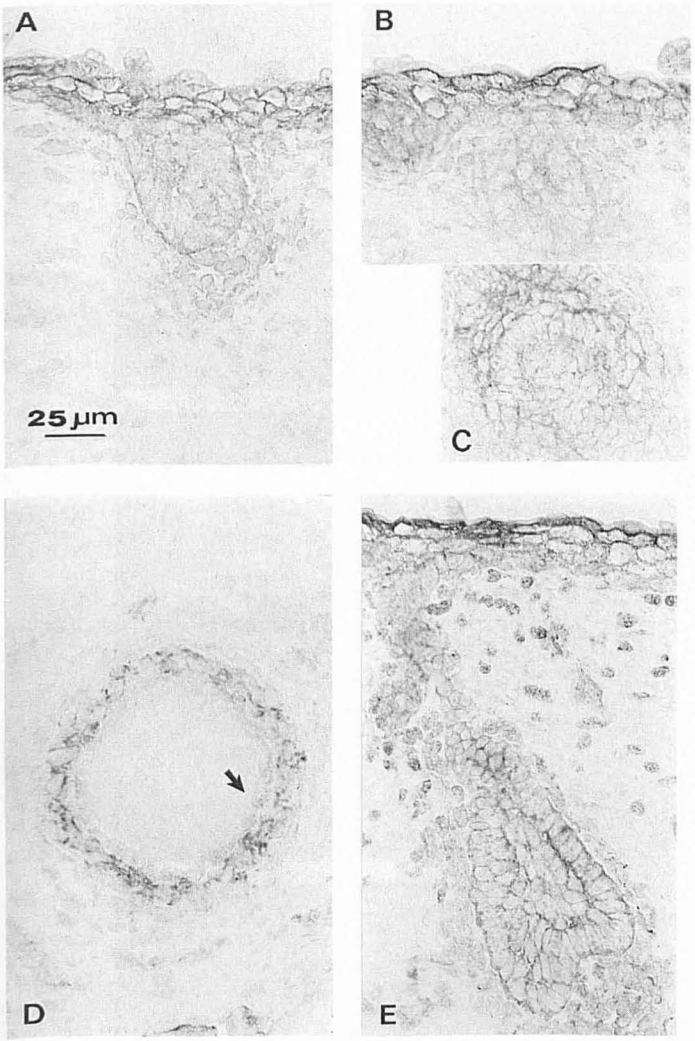


Figure 3. Amphiregulin immunostaining is modulated in fetal skin at 81 d (*A*), 96 d (*B,C,D*), and 103 d (*E*) EGA. The epidermis is reactive for amphiregulin, although hair buds have relatively decreased amphiregulin (*C*). The dermal mesenchymal cells surrounding the hair buds exhibit more staining compared to other dermal cells. In the bulbous hair peg there is increased staining superficial to the bulb region (*C*). Amphiregulin is strongly stained in the dermal blood vessel but mostly absent from the endothelial cells (*arrow, D*). At 103 d EGA (*E*) cells strongly reactive for amphiregulin include the outermost keratinocytes, cells in the papillary dermis, hair follicle keratinocytes, and surrounding mesenchymal cells. The periderm is nonreactive.

papilla, nerve **(Fig 5*C*)**, adnexal sheath, and dermal vasculature **(Fig 5*E*)**, showed strong punctate nuclear staining.

There was cytoplasmic and nuclear staining for amphiregulin in all viable layers of the epidermis of newborn foreskin **(Fig 6*A*)**. Dermal connective tissue cells were generally less reactive than those of fetal dermis, although there was strong retention of smooth muscle staining **(Fig 6*C*)**. In contrast, adult skin showed epidermal staining of only a few spinous keratinocytes with predominantly random nuclear localization **(Fig 6*B*)**. Dermal structures and connective tissue cells were mostly nonreactive, but there was often focal staining within the inner root sheath and occasionally other structures of the follicle such as the sebaceous lobules and within blood vessels and smooth muscle cells **(Fig 6*D,E*)**. Sections pre-fixed in 2% paraformaldehyde exhibited predominantly cytoplasmic staining similar to that of the ethanol fixed sections, indicating no redistribution of antigen from alcohol fixation.

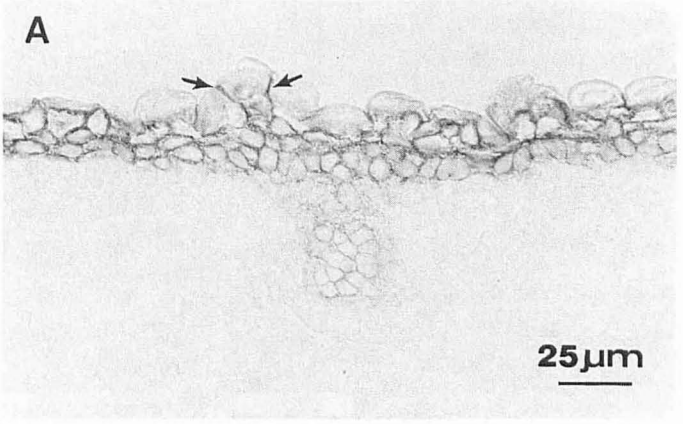


Figure 4. Immunostaining for the epidermal growth factor receptor in fetal skin at 87 d EGA contrasts with that of amphiregulin. The epidermis is reactive for the receptor, and note its staining along the basal and lateral borders of the periderm cells (*arrows*).

Amphiregulin Reactivity Is Nonrandomly Localized in Cultured Neonatal Keratinocytes Amphiregulin expression was characterized in neonatal keratinocytes cultured in serum-free medium without exogenous growth factors. The protein detection was by immunofluorescence with the fluorescein isothiocyanate–conjugated secondary antibody. In low-density cultures, keratinocyte colonies segregated amphiregulin to specific topographic sites **(Fig 7)**. Cytoplasmic staining was the predominant pattern, with increased immunoreactivity of outer membranes at the perimeter of small colonies; juxtaposed membranes between keratinocytes in the interior of colonies were usually either less intensely stained or were nonreactive **(Fig 7*A*)**. Throughout the colonies, the nuclei showed heterogeneous patterns of both punctate and diffuse staining **(Fig 7*A,B*)**, with random perinuclear reactivity.

Subcellular localization of amphiregulin was further characterized by scanning laser confocal microscopy **(Fig 7*C–H*)**. Amphiregulin was absent from the ventral surfaces of keratinocytes in contact with the substratum; rather, it was distributed to the dorsal plasma membranes and the lateral perimeter of the cells **(Fig 7*E,F*)**. Immunostaining with antibody to type IV collagen demonstrated antibody accessibility to all cell compartments (not shown). Staining of membranes was coarse and variably granular **(Fig 7*C–F*)**. The intracytoplasmic patterns were usually granular, but filamentous or tubular patterns were noted. Keratinocytes undergoing cytokinesis had coarser aggregates, and occasionally there were plate-like arrays of staining with the long axes perpendicular to the mitotic spindle. Confocal images of sections through the nuclei revealed heterogeneous punctate amphiregulin staining **(Fig 7*E*)**. Intranuclear staining was less dense than that of the cytoplasm; some cells had almost no nuclear staining **(Fig 7*F*)**, whereas postmitotic nuclei (e.g., binucleate cells prior to cytokinesis) were more reactive. Higher density keratinocyte cultures were less reactive (compare panels *D* and *F* with *C* and *E* in **Fig 7**). Some peripheral membrane staining was retained, but there was more perinuclear and less intranuclear localization.

DISCUSSION

Evidence in support of a central function for the epidermal growth factor-like ligands in keratinocyte function has come, in part, from antibody-blocking studies that indicate more than 90% of autocrine growth of keratinocytes is mediated through the epidermal growth factor receptor [5]. Immunolabeling studies have shown restricted localization of the receptor to the basal and immediately suprabasal keratinocytes in adult skin but in all epidermal layers of fetal skin, which support the hypothesis that its expression correlates with cell proliferation [18,19]. In contrast, epidermal growth factor localizes

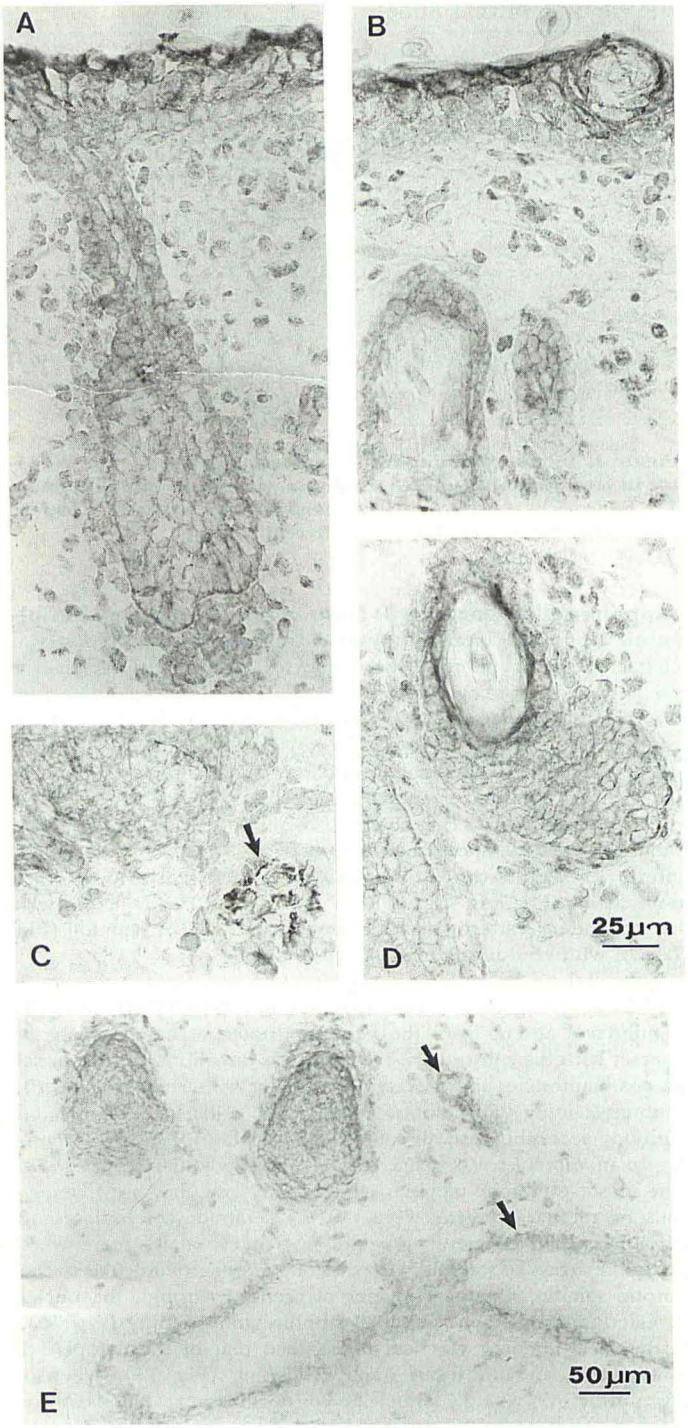


Figure 5. Immunostaining patterns for amphiregulin further evolve in fetal skin at 115 d (*A*) and 125 d (*B–E*) EGA. Amphiregulin staining is increased in many cells throughout fetal skin, notably in the bulbous hair follicle (*A*), the hair canal and bulge (*B,D*), and dermal papillae and bulb (*C,E*). A nerve fiber is strongly reactive for amphiregulin (*arrow, C*), as are blood vessels (*arrows, E*).

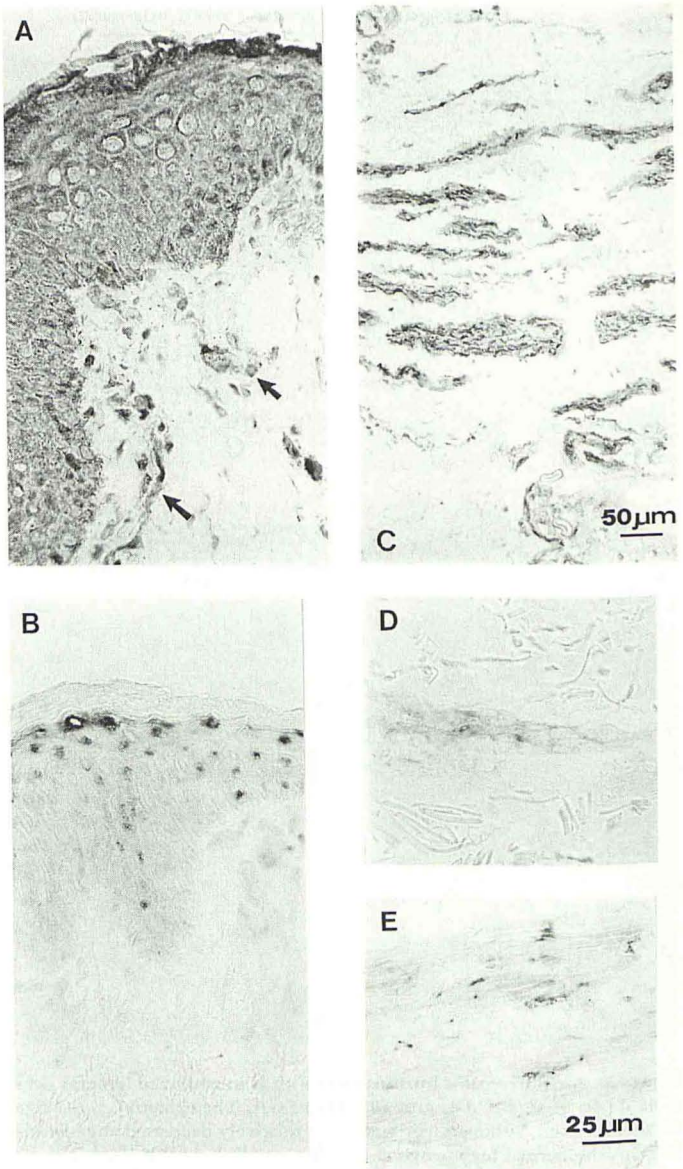
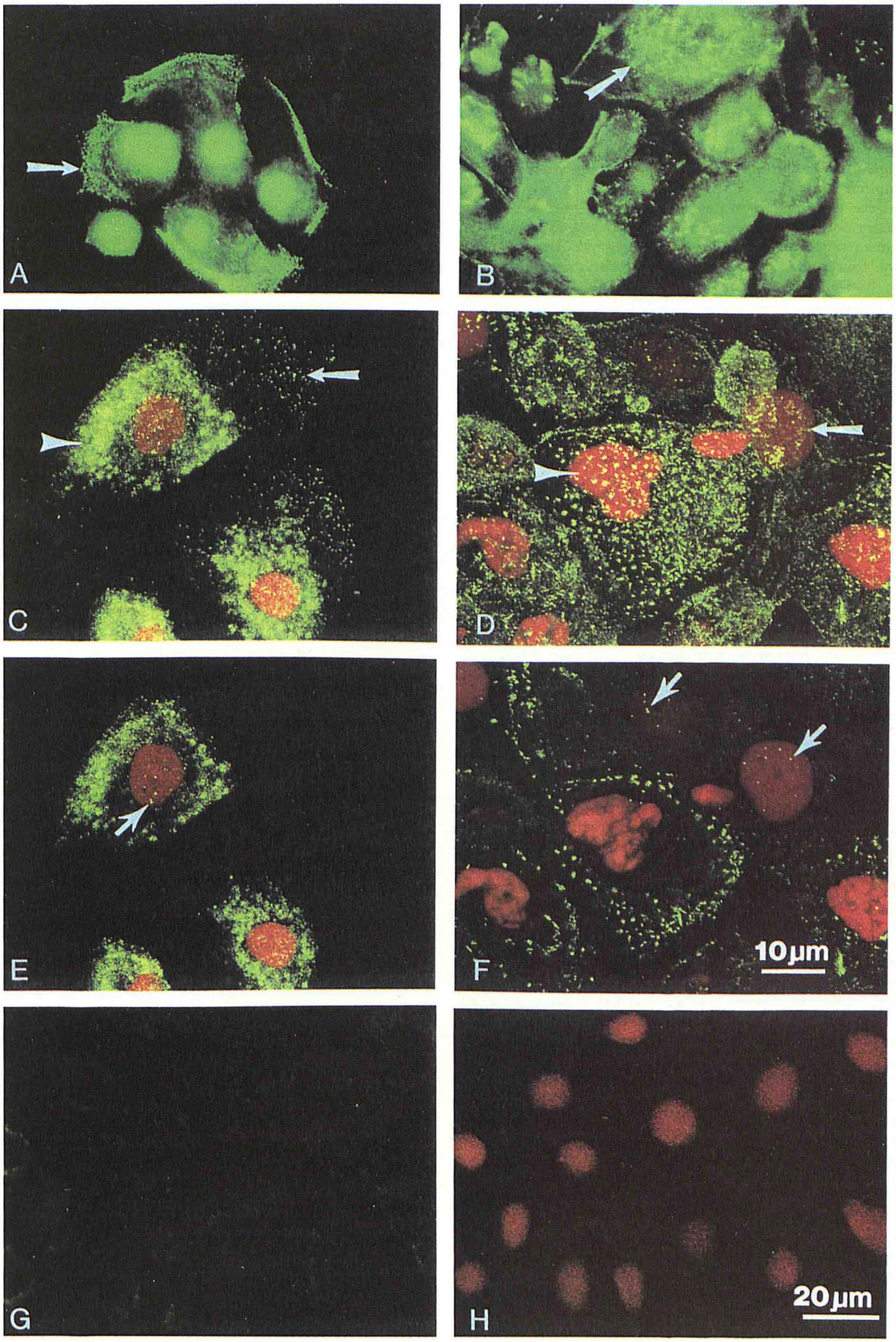


Figure 6. Amphiregulin immunostaining patterns are different in newborn (*A,C*) and 70-year–old adult (*B,D,E*) skin. Newborn foreskin epidermis, dermal cells, and capillaries (*arrows*) are strongly stained (*A*), as are bundles of smooth muscle (*C*). In adult skin individual cells of the epidermis are reactive (*B*), and some dermal blood vessels are stained (*D*). Cells of the arrector pili muscle are also reactive (*E*).

predominantly to the suprabasal cell layers [19]. Although the basis for the differential localization of the receptor and cytokine is not clear, it does suggest a role for other ligands in epidermal growth.

Amphiregulin is one of several cytokines that are structurally homologous to the epidermal growth factor. Among that family of factors, its affinity for heparin-like glycosaminoglycans is shared only by heparin-binding epidermal growth factor [20,21]. The

Figure 7. Immunostained amphiregulin was localized in keratinocyte cultures by epifluorescence microscopy (*A,B*) and scanning laser confocal microscopy (*C–H*). Subconfluent keratinocytes had punctate staining for amphiregulin at the outer (leading) edge of cells in small clusters (*A, arrow*). In larger colonies (*B*) amphiregulin staining patterns were heterogeneous; dense staining is indicated (*arrows*). By confocal microscopy, composite images (*C,D*) are compared to their respective 1-micron slices at the level of the nuclei (*E,F*), which have been stained with propidium iodide and imaged in the red spectrum. Subconfluent (*C,E*) and confluent (*D,F*) conditions both show punctate amphiregulin reactivity in the nuclei and coarse granular staining of the cytoplasm, as indicated. There was minimal background fluorescence in control cultures without primary antibody (*G*, FITC channel), with nuclei indicated in the red channel (*H*).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importance of heparin-binding autocrine factors in keratinocyte growth emerged from the observation that heparin-like glycosaminoglycans strongly inhibit keratinocyte growth; subsequent studies implicated amphiregulin to be a target of that effect [2,3,5]. Exogenous recombinant amphiregulin is an effective growth-stimulating factor for cultured keratinocytes, with a potency similar to that of epidermal growth factor [5]. Moreover, blocking antibody studies have suggested that most of keratinocyte autocrine growth under serum-free conditions is attributable to endogenous production of amphiregulin [5].

Because the evidence indicates amphiregulin to be a major growth factor for keratinocytes, its transcriptional expression in several human skin diseases has been characterized. Amphiregulin mRNA was found to be expressed at low levels in normal human epidermis, but expression increased in psoriatic lesions and in cutaneous squamous cell carcinomas [15–17]. Augmented synthesis of amphiregulin could partly mediate the hyperplastic epidermal phenotype of hyperproliferative skin disorders.

Our observations extend current information to the detection of immunostained amphiregulin during fetal skin morphogenesis and in cultured keratinocytes. We found that the staining patterns evolved during embryonic and fetal development. In the embryonic stage, the periderm was strongly reactive, but at later gestational ages reactivity declined. The epidermal growth factor receptor is expressed at low levels in the periderm and is concentrated at the basal and lateral surfaces at all developmental stages [18], indicating a partially discordant distribution compared with that of amphiregulin. During the complex bleb stage of periderm regression after 15 weeks EGA, amphiregulin was stained along the basal cytoplasm of the periderm. The mechanism of periderm regression has been attributed to apoptosis [22]; according to that model, our data indicate a reciprocal relationship between expression of amphiregulin and apoptosis in the periderm.

Keratinocyte expression of amphiregulin was also modulated during gestational development and postnatally. Embryonic basal keratinocytes were reactive; with later formation of intermediate epidermal cell layers by 12 weeks EGA, the reactivity became stronger in the suprabasal keratinocytes. By 15 weeks EGA, the most superficial keratinocyte layer, which remains covered by periderm, stained intensely throughout the cells, but the deeper keratinocytes were less reactive. In newborn foreskin, all viable epidermal layers were reactive, whereas staining was barely detectable in adult skin, except for random spinous keratinocyte nuclear staining and variable follicular reactivity (see below). These patterns can be partly explained by the correlation of its expression with proliferative activity, but other features of its distribution, such as the random nuclear localization, suggest additional cellular functions.

At all stages of follicle formation, amphiregulin was stained in both epithelial and mesenchymal cell components of the folliculosebaceous units. After 16–20 weeks gestation, for example, the hair follicle, sebocytes, bulge region, and hair matrix were all reactive. The inner root sheath retained staining into the adult period, whereas retention by the other follicular components was variable and often nonreactive. In contrast, at the early germ and peg stages the epidermal growth factor receptor is essentially absent (or masked) [18]. Follicular morphogenesis has been characterized as a regulated program of spatially and temporally restricted, inductive interactions between specialized epidermal cells and mesenchyme [23]. The observation that multiple cellular constituents of the embryonic follicle were reactive for amphiregulin implicates the cytokine as a candidate morphogen in follicle development. Considered with prior data showing low epidermal growth factor receptor expression early in follicle morphogenesis [18], our data also suggest that amphiregulin may in part act independently of that receptor. The persistence of the inner root sheath staining in adult skin may reflect additional functions in the mature follicle.

Our data, in conclusion, indicate that expression of amphiregulin is developmentally regulated in the epithelia and mesenchyme of human skin during morphogenesis. The distinct follicular reactivity commencing at the primordial germ stage further suggests an active and regulatory role for amphiregulin in follicle development. The selective segregation to the perimeter of cell colonies and to nuclei in keratinocyte cultures supports the hypothesis that it functions as an autocrine growth factor. Further study is needed to identify the mechanisms by which amphiregulin influences the cellular phenotype.

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